

REMARKS

I. Introduction

Receipt of a final office action dated March 9, 2006, is acknowledged. In the action, claims 23-24 are rejected as allegedly obvious over US Patent No. 5,298,420 ("420 patent"), in view of Goto *et al.*, *Blood*, 84(6):192201930 (1994) ("Goto *et al.*"). Additionally, claims 13 and 15-22 are rejected for failing to meet the written description requirement.

Applicants respectfully request reconsideration of the present application in view of the reasons that follow.

II. Amendments to the Claims

In the office action, no new claims have been added or amended. Upon entry of this amendment, claims 13 and 15-24 will be under examination.

A detailed listing of all claims that are, or were, in the application, irrespective of whether the claims remain under examination in the application, is presented, with an appropriate defined status identifier.

III. Rejection of the Claims Under 35 U.S.C. 103(a)

Claims 23-24 are rejection as allegedly obvious over the '420 patent, in view of Goto *et al.* In particular, the claims are rejected because

[i]t would have been *prima facie* obvious to one of ordinary skill in the art . . . to perform a method of inhibiting B lymphocyte activation (by killing the lymphocyte) for the treatment of an autoimmune disease, comprising administering a monoclonal antibody, as taught by the ['420] patent, employing the humanized monoclonal antibody HM1.24 which binds SEQ ID NO:1, as taught by Goto, T., et al. as the specific monoclonal antibody.

Office action at 3. Applicants respectfully disagree.

To establish a *prima facie* case of obviousness, there must be: (1) some suggestion or motivation to modify the reference or to combine reference teachings, (2) a reasonable expectation of success, and (3) when combined, a teaching or suggestion of all the claim

limitations in the prior art references. *See* MPEP §2143 (Aug. 2001). “Both the suggestion and the reasonable expectation of success must be founded in the prior art, not in the applicant’s disclosure.” *In re Vaeck*, 947 F.2d 488, 493 (Fed. Cir. 1991).

Goto describes use of an anti-HM1.24 antibody to identify a marker of late stage B-cell maturation but does not teach or suggest administering the antibody to inhibit lymphocyte proliferation. This deficiency, however, is not taught in the ’420 patent.

The ’420 patent demonstrates that immunosuppression can be established by the use of antibodies which bind epitopes that are specific extracellular regions in 5 membrane-binding types of Ig molecules. See the ’420 patent at col. 3, lines 10-30. In other words, the ’420 patent describes that migis- δ and - μ antigens are useful because all B cells pass through a stage where they produce IgM and IgD, and that migis- γ is useful because a large amount of IgG is present in an organism and any antibody whose epitope is migis- γ is useful to any IgG subclass since the sequence of migis- γ in 4 subclasses of IgG are the same. **The teachings in the ’420 patent, however, do not extend beyond targeting migis epitopes** and not all B cell epitopes are migis epitopes. And since the presently claimed invention does not recite the use of the membrane-binding type of Ig molecule-binding antibody, there is no motivation to combine the cited references with a reasonable expectation of successfully employing the antibody recited in the claims in a method for inhibiting lymphocyte activation.

Furthermore, as previously stated, the ’420 patent describes cytotoxic activity as a preferred embodiment of immunosuppression (see ’420 patent at col. 6) but doesn’t describe methods other than cytotoxic activity as a means of immunosuppression. But as stated in applicant’s last response, the presently claimed invention does not recite the use of such cytotoxic activity.

Therefore, for at least these reasons, the obviousness rejection should be withdrawn.

IV. Rejection of the Claims Under 35 U.S.C. 112

Claims 13 and 15-22 are rejected under 35 U.S.C. § 112, first paragraph for allegedly failing to meet the written description requirement. In particular, the claims are rejected

because “the specification provides insufficient evidence that the antibody employed in the claims could bind T lymphocytes as is required by the claimed method.” Office Action at 3-4. Applicants respectfully traverse this ground for rejection.

Goto et al. describes using peripheral blood obtained from a healthy person and stimulating it with PWM. PWM is a creatine molecule often used as a mitogen which stimulates immunocytes, similarly to Con A and HAP as used in the present invention (for example, see, the enclosed Clinical and Diagnostic Laboratory Immunology, 1998, 5, pp. 105-113, APPENDIX A). But according to the result obtained using the PWM stimulated peripheral blood, expression of the HM1.24 antigen in the peripheral cells was not observed until 10 days after stimulation. See Goto at page 1926, right column and Fig. 3, at page 1927. The applicant considers that Fig. 2 does not refer to T-cells.

It was later revealed, however, that the binding difference could also have been caused by a difference in the threshold setting of FACS used in the analysis. In fact, afterwards, the molecule shown as SEQ ID No: 5 was internationally recognized as a cell marker, and an authorized expression profile was described in literature (see, the enclosed Cellular Immunology, 2005, 236 pp. 6-16, APPENDIX B). According to the expression profile, it is clear that both inactivated T-cells and activated T-cells express the HM1.24 antigen (see Table 5 on page 11).

Lastly, the tested cells in the Goto reference were not concentrated by purification as T-cell fraction while in the present invention, the concentration was carried out. Therefore, the sensitivity in the Goto reference was too low for detection.

Therefore, for at least these reasons, the written description rejection should be withdrawn.

CONCLUSION

Applicant believes that the present application is now in condition for allowance. Favorable reconsideration of the application as amended is respectfully requested.

The Examiner is invited to contact the undersigned by telephone if it is felt that a telephone interview would advance the prosecution of the present application.

The Commissioner is hereby authorized to charge any additional fees which may be required regarding this application under 37 C.F.R. §§ 1.16-1.17, or credit any overpayment, to Deposit Account No. 19-0741. Should no proper payment be enclosed herewith, as by a check or credit card payment form being in the wrong amount, unsigned, post-dated, otherwise improper or informal or even entirely missing, the Commissioner is authorized to charge the unpaid amount to Deposit Account No. 19-0741. If any extensions of time are needed for timely acceptance of papers submitted herewith, Applicant hereby petitions for such extension under 37 C.F.R. §1.136 and authorizes payment of any such extensions fees to Deposit Account No. 19-0741.

Respectfully submitted,

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Immune Function in Healthy Adolescents

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In the present study, we examine immunological functioning in normal healthy African-American and Latino/Latina adolescents recruited from an inner-city high school and an inner-city clinic. A battery of tests was performed with enumerative and functional measures which encompassed both innate and adaptive immunity. We found immune differences related to age, gender, and race on both the enumerative and the functional immune measures. This data expands the available body of information concerning normal immunity in healthy adolescents.

The immune system has most often been studied in relation to disease, and much of the normative data has been compiled by considering various control groups in different studies. Further, while there is considerable data on immune parameters in Caucasian adults, there is much less data available for other age groups, such as adolescents, and for other ethnic groups.

Some researchers have reported normative immune data when studying disease processes in adolescent populations (3, 11, 31, 35). However, since the focus of these studies was not normal subjects, the description of the normal controls as well as the small number of subjects limit the usefulness of this work for providing normative data. More recent studies have begun to provide some selective normative immunological data with normal, healthy adolescent subjects. For example, in one study enumerative (cell phenotype) data from 112 predominantly Caucasian (74%) healthy adolescents (ages 12 to 19) (40) was examined, while in another study the functioning of polymorphonuclear leukocytes in 58 children (aged 6 months to 15 years) (15) was examined. Although these studies are limited by the fact that they were focused on a single immunological variable, they are a useful beginning in understanding normal adolescent immune status.

The present study was part of a longitudinal study of behavior, mood, and immunity in inner-city minority adolescents. We evaluated 206 healthy African-American and Hispanic adolescents, utilizing fresh blood cells and a battery of immune assessments which provides data on enumerative and functional immunological measures. The measures included total leukocyte count, counts of both granulocytes and lymphocytes, and counts of subsets of the lymphocyte populations, including those shown to have implications in certain disease states such as human immunodeficiency virus illness. The functional measures that were chosen involve *in vitro* assays only. Therefore, no exposure to antigen or other invasive procedure was necessary. This was thought to decrease subject risk and increase participation of subjects.

This data is characterized in terms of the relationships to age, gender, and race within this selected population of healthy inner-city youth. Normative data from this type of under-studied population has become increasingly important with regard

to immune-related diseases. Since the advent of diseases such as AIDS, the need for knowledge of immunity in adolescents as well as the need for following the progression of this or other disease states in this population has increased.

MATERIALS AND METHODS

Subjects. This study was approved by the Institutional Review Board of UMDNJ-New Jersey Medical School. Informed consent from subjects 18 years of age and informed assent from subjects under 18 years of age with informed consent from a parent or guardian were obtained. A total of 331 adolescents ranging from 12 to 18 years of age participated in the present study. All subjects were recruited as part of a project assessing behavior, immunity, and health. Two hundred thirteen subjects were randomly recruited from a local public high school. One hundred eleven consecutive adolescents who were attending an adolescent medicine clinic for a routine physical examination or follow-up for a minor medical condition were also recruited. Seven subjects were peer referrals. All psychosocial (e.g., age and race) and substance use (e.g., alcohol and tobacco) data were obtained in an interview format.

A medical history, review of systems, and vital signs were obtained. Potential subjects were excluded if they had chronic diseases likely to have substantial effects on immunity (e.g., neoplastic, endocrine, or immune disorders) or if they were taking medications with known immunologic effects. Subjects with acute infections were deferred from study until their symptoms were resolved. Subjects presenting with other medical conditions, such as recently resolved minor infections, or with other past disorders with possible immune effects (e.g., asthma) were studied but not included in these analyses. The occasional adolescent with clinically apparent mental retardation, significant neurologic deficits, schizophrenia, or substance abuse or dependence disorders was excluded.

Medical group evaluation. Each subject was screened by a trained research assistant with the Health Symptom Checklist (18). Vital signs were collected at the time of venipuncture. All medically relevant data was reviewed by the physicians (J.A.B., S.J.S., and B.R.D.) who made the final consensual determination of the subjects' medical status. Subjects were classified into one of the following three medical groups: (i) healthy (subject had no medical problems and was not taking any medication); (ii) minor medical problem (subject had mild medical symptoms such as a cough or runny nose, had had fever within the past week [venipuncture was deferred in subjects with current fever], or had taken medication for a cold within the past 2 weeks); or (iii) medical problem (subject was found to have more significant chronic or current medical problems considered likely to be associated with altered immunity). This last group consisted mainly of asthmatics who, in the past year, had had an asthmatic attack or utilized antiasthmatic medication and of adolescents with a history of a recent infection requiring antibiotic therapy. Of the total 331 adolescents studied, 206 were classified in the healthy group prior to the analysis of any data. Only the subjects in the healthy group were included in the analyses to be described.

Immunological evaluation. All assays were carried out blind to the subjects' medical status. Blood samples were collected in a heparinized syringe (preservative-free heparin). All the results herein were obtained from the same venipuncture for each subject. Total leukocyte and differential counts were performed by standard techniques. Phenotype analysis of lymphocytes, monocytes, and granulocytes was performed with heparinized whole blood. Mononuclear cells were separated from whole blood by centrifugation on a Ficoll-Hypaque gradient. These cells were used to assess mitogen-induced lymphocyte stimulation and natural killer (NK) cell function. Additionally, granulocyte function was

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determined from granulocytes which were isolated from the remaining erythrocytes by a percoll gradient.

Cell phenotypes. Cell phenotypes were assessed by flow cytometry with the Epics Profile Plus (Coulter Immunology, Hialeah, Fla.). All monoclonal antibodies were directly conjugated and obtained from Coulter Immunology. The antibodies were all used in concentrations of 10 μ l per 0.10 ml of whole blood. Q-prep technology was then utilized to process the samples for cytometry. Briefly, 50 μ l of heparinized whole blood was added to 200 μ l of the appropriate antiserum or antiserum combination. Samples were incubated on ice for 45 min, and then the erythrocytes were lysed and the preparation was suspended in paraformaldehyde and sheath fluid. The monoclonal antibodies utilized for the following cells are indicated in parentheses: lymphocytes (CD45) (KCS6-fluorescein isothiocyanate), monocytes (CD14) (MO2-RDI), and granulocytes (CD11b) (forward and side scatter, no antibodies), T cells (CD3), B cells (CD19), NK cells (CD56), T helper cells (CD4), T-cell suppressor/cytotoxic T cells (CD8), T-cell suppressor inducer (CD4 plus CD45RA), T helper cell inducer (CD4 plus CD29), and activated T cells (CD3 plus HLA-DR, DP, and DQ).

Appropriate filter combinations were used to simultaneously measure emissions from fluorescein isothiocyanate and phosphatidylethanolamine. Gates were selected with forward and 90°-angle light scatter to select the cell population of interest. A minimum of 1,000 cells were included in each analysis. The color compensation was performed by examining the percentage of P11 (green) seen in the F12 (red) and, conversely, the percentage of P12 seen in the F11 channel. For this determination, CD4-FITC and CD8RD were utilized as staining control lymphocytes. Roughly, F11 had 0 to 3% of P12, while P12 had 3 to 5% of P11.

Cell viability was determined by trypan blue exclusion. Cells were counted in a hemacytometer and viability was determined by the percentage of cells that did not take up the dye. Cells were then washed and resuspended in RPMI medium. Target K562 cells were maintained by passaging every 2 to 3 days. Cell viability was always >98%. On the day of assay, target cells are collected, washed, and labeled with 500 μ Ci of 51 Cr for 2 h at 37°C. Target cells are then washed three times, and 10^6 cells are plated in microtiter plate wells. The NK cells are isolated from the whole blood as described above and prepared in 3 dilutions in RPMI with 15% normal human serum. The final concentrations of NK cells are 25×10^4 , 50×10^4 , and 100×10^4 . This provides three effector-to-target ratios (25:1, 50:1, and 100:1). The mixture of target cells and NK cells is then incubated for 4 h at 37°C. The microtiter plates are then centrifuged, and the supernatant from each well is assayed for 51 Cr activity. The NK data is presented as percentage of specific cytotoxicity (see Data Analyses).

Granulocyte activity. Granulocyte activity was assessed by examining both the phagocytic and killing ability of granulocytes according to the method described by Weir (41), with the following modifications. After separation of the lymphocytes from the peripheral blood by a Ficoll-Hypaque gradient, the granulocytes were separated from the erythrocytes by a percoll gradient. The granulocytes were incubated with opsonized *Staphylococcus aureus* for 20 min. The incubation mixture was centrifuged, and the unopsonized *S. aureus* was washed out with cold RPMI medium. Granulocytes were resuspended in RPMI, and aliquots were prepared for further processing. One aliquot was processed immediately to assess the phagocytic ability of the granulocytes. Two aliquots were incubated at 37°C to assess killing ability, and two aliquots were incubated on ice as controls. At time 0, 1-h, and 2-h postincubation with *S. aureus*, the granulocytes were lysed with 0.5% bovine serum albumin in distilled water and plated on blood agar plates. The plates were incubated at 37°C for 24 h. The number of colonies was counted; each represents an ingested *S. aureus* bacterium. Differences between the numbers of colonies at 37 and 0°C represent specific killing of bacteria.

Data analyses. Data were first examined descriptively for distribution, means, and variance. Multivariate models, defined a priori, were tested by regression analysis. For each analysis of the enumerative measures, we examined the contribution of age, gender, or race, controlling for the other two variables and for the total leukocyte count (WBC) (simultaneous, not hierarchical), with number of leukocytes covaried. For the mitogen response, NK cell activity, and granulocyte activity, WBC was not included in the model. All tests were two-tailed.

The mean of the highest two responses to each of the mitogens and the mean of the two highest effector-to-target killing ratios for NK cell activity were utilized to form dependent variables for these regression analyses. Also, while we examined the NK cell activity results both as specific killing and as the number of lytic units, we have presented the data as percentage of killing for several reasons. First, the use of specific killing was closer to the raw data and less

TABLE 1. Demographic data for subjects in this study

Characteristic	Female (n = 106)	Male (n = 100)	Total sample (n = 206)
African-American	83	94	177
Latino/Latina	17	12	29
Age (mean \pm SD) (yr)	15.8 \pm 1.6	15.8 \pm 1.8	15.8 \pm 1.7

manipulated and allows direct inspection of the levels of specific cytotoxicity. Secondly, not all of our data fits the assumptions required for lytic unit transformation and requires either the dropping of particular data points, the dropping of particular cases, or the truncation of the data. None of the options seems preferable to the presentation of the data as percentage of killing.

The granulocyte activity assay was initiated in the latter part of the study. However, sufficient numbers of adolescents were studied to permit analysis of this data (n = 96).

RESULTS

Demographics. As shown in Table 1, there were 100 males and 106 females in this sample of healthy subjects (n = 206). The sample was comprised of 177 (83 female) African-Americans and 29 (17 female) Latinos/Latinas. The mean age \pm standard deviation for the entire sample was 15.8 \pm 1.7 years. The mean age of the males was 15.75 \pm 1.81, and that of the females was 15.83 \pm 1.59. The age distribution is presented as a function of gender in Fig. 1. The age, gender, and race distributions provided sufficient power to allow us to meaningfully assess their contributions to the variance of the immune measures.

Immune measures. The findings for the entire sample are presented in Table 2 and Fig. 2 through 4. The enumerative measures are presented in Table 2 both as absolute number of cells present and as a percentage of the total WBC. The dose-response curves for each of the three mitogens are shown in Fig. 2. In Fig. 3, the dose-response curve of NK cell activity at the three effector-to-target ratios utilized is shown. In Fig. 4, the killing of *S. aureus* (at 37°C and on ice) by granulocytes is presented.

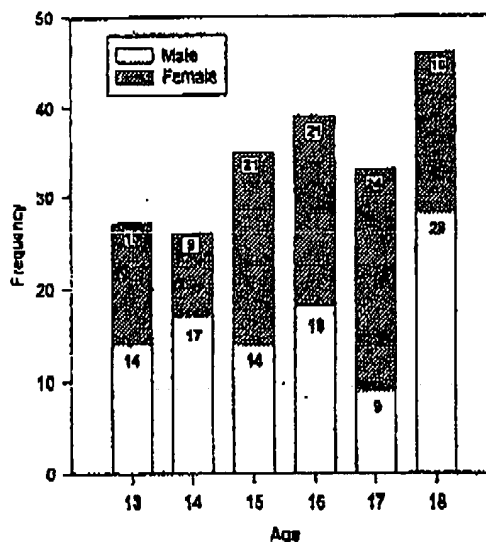


FIG. 1. Age distribution as a function of gender (n = 206).

TABLE 2. Totals for peripheral leukocyte measures for entire sample

Variable	No. of cells (10^6 /ml (mean \pm SD)	% Leukocytes (mean \pm SD)
Leukocytes	7.11 \pm 2.82	
Lymphocytes	1.57 \pm 0.54	37.25 \pm 9.71
Granulocytes	2.63 \pm 1.18	55.39 \pm 10.35
Monocytes	0.29 \pm 0.12	6.68 \pm 2.98
T lymphocytes	1.22 \pm 0.46	76.55 \pm 8.48
B lymphocytes	0.23 \pm 0.13	15.29 \pm 5.94
CD4 ⁺ lymphocytes	0.69 \pm 0.27	42.98 \pm 7.38
CD8 ⁺ lymphocytes	0.33 \pm 0.16	20.95 \pm 5.46
Helper-to-suppressor ratio		2.31 \pm 0.07
CD29 ⁺ lymphocytes (inducers of help)	0.29 \pm 0.15	17.44 \pm 6.63
CD45RA ⁺ lymphocytes (inducers of suppression)	0.34 \pm 0.17	21.12 \pm 6.67
CD56 ⁺ lymphocytes (NK cells)	0.07 \pm 0.08	4.19 \pm 4.32
HLA-DR ⁺ lymphocytes (activated T cells)	0.10 \pm 0.06	5.87 \pm 3.79

Effects of age, gender, and race. We examined the contributions of age, gender, and race on the various immune parameters.

Age effects. (i) Cell phenotypes. WBC was positively correlated with age (partial $r = 0.25$, $P < 0.001$) (Fig. 5). Age also contributed to the percentage of CD29⁺ cells ($F = 3.25$, $P < 0.002$) (Fig. 6), with older subjects having a higher percentage; to the number ($F = 2.31$, $P < 0.03$) and percentage ($F = 2.14$, $P < 0.04$) of B cells, with younger subjects having higher values; and to the percentage of NK cells ($F = 2.34$, $P < 0.03$), with older subjects having higher percentages.

(ii) Mitogen-induced lymphocyte stimulation. For the proliferation assays, there were no significant effects of age on the mean lymphocyte responses to the mitogens ConA ($F = 0.56$, $P > 0.10$), PWM ($F = 1.17$, $P > 0.10$), or PHA ($F = 1.60$, $P > 0.10$).

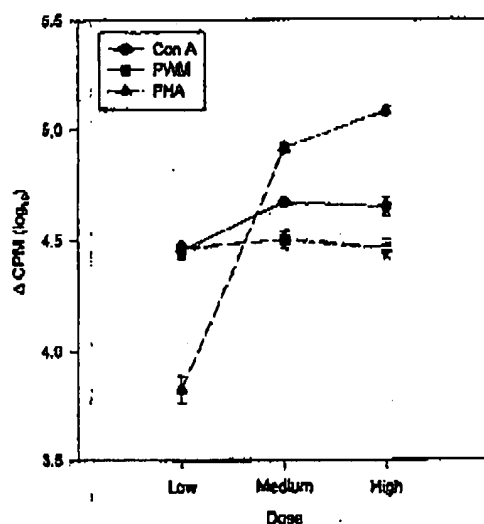


FIG. 2. Dose-response curves of the lymphocytes to each of the three mitogens (ConA, PHA, and PWM). Data has been log transformed and is presented as the mean \pm the standard error of the mean ($n = 206$).

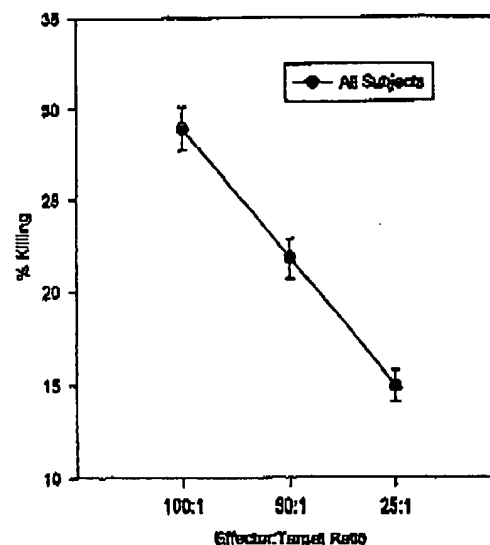


FIG. 3. Dose-response curves of the NK cell killing at the three effector-to-target ratios. Data is presented as the mean \pm the standard error of the mean ($n = 206$).

(iii) NK cell activity. There were no relationships between age and mean NK cell cytotoxicity ($F = 0.62$, $P > 0.10$). When the number of NK cells was also controlled, this relationship did not change substantially.

(iv) Granulocyte activity. This assay was initiated in the latter part of the study. However, sufficient numbers of adolescents were studied to permit the analysis of this data ($n = 96$). As seen in Fig. 7, there was a significant decrease in the phagocytosis of *S. aureus* with increasing age ($F = 3.33$, $P < 0.01$). In addition, after peaking when the subjects were age 14,

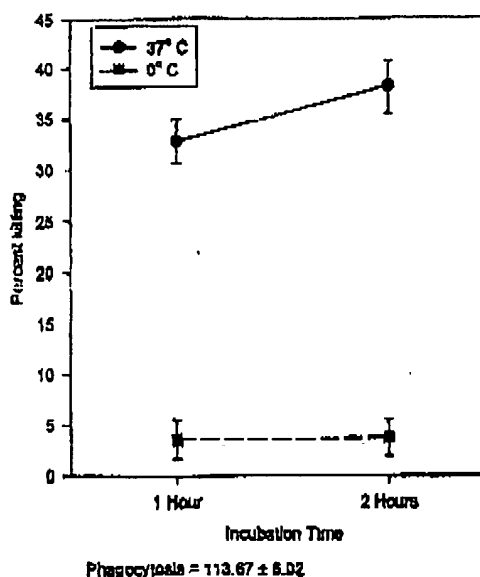


FIG. 4. Killing of *S. aureus* by polymorphonuclear granulocytes. Data is presented as the mean \pm the standard error of the mean ($n = 206$).

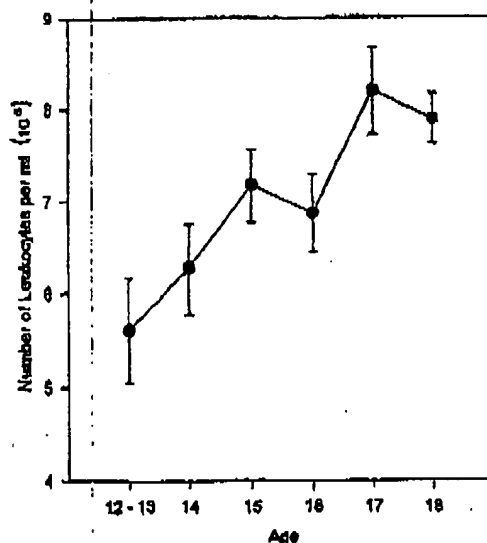


FIG. 5. WBC (mean \pm standard error) as a function of age; partial r , 0.25; P , <0.001.

the percentage of bacteria killed at 1 h ($F = 2.13$, $P < 0.04$) and 2 h ($F = 2.95$, $P < 0.005$) declined with age (Fig. 8).

Gender effects. (i) Cell phenotypes. As shown in Table 3, no difference in total WBC or in numbers of lymphocytes, granulocytes, monocytes, or NK cells was found between males and females. There was a significantly lower percentage (but not number) of T cells in males than in females ($F = 5.85$, $P < 0.0001$). The number of B cells ($F = 3.43$, $P < 0.0009$) was higher in males, as was the percentage ($F = 2.14$, $P < 0.04$). There were significantly lower numbers of CD4⁺ cells in the male adolescents than in the female adolescents ($F = 2.24$, $P < 0.03$). Similarly, there was a lower percentage of CD4⁺ cells among males than among females ($F = 5.85$, $P < 0.0001$).

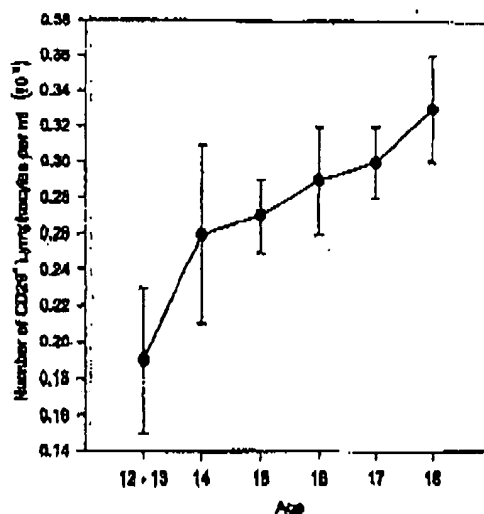


FIG. 6. Percentage of CD29⁺ (inducer of help) as a function of age (mean \pm standard error); partial r , 0.31, P , 0.001.

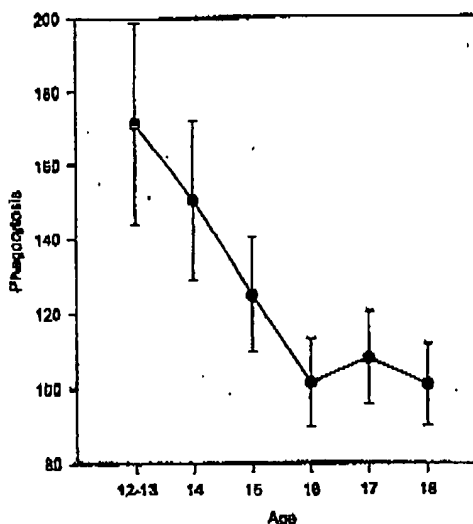


FIG. 7. Number of *S. aureus* cells phagocytosed as a function of age ($P < 0.04$). Data is presented as the mean \pm standard error of the mean ($n = 206$). The 12- and 13-year-old subjects were combined due to the small number of subjects in these two groups.

Additionally, the percentage but not the number of CD29⁺ cells (inducers of help) was lower among males than among females ($F = 2.54$, $P < 0.02$). Further, the helper-to-suppressor ratio was higher in females than in males ($F = 2.44$, $P < 0.02$). No other differences in numbers or percentages of cells were found.

(ii) Mitogen-induced lymphocyte stimulation. For the proliferation assays, there were no significant effects of gender on the mean lymphocyte responses to ConA ($F = 0.40$, $P > 0.10$), PWM ($F = 1.50$, $P > 0.10$), or PHA ($F = 1.27$, $P > 0.10$).

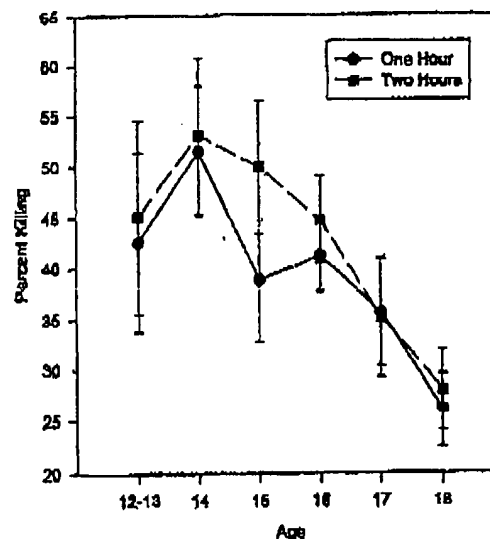


FIG. 8. The percentage of *S. aureus* cells killed as a function of age ($P < 0.01$). The data is presented as the mean \pm standard error of the mean ($n = 206$). The 12- and 13-year-old subjects were combined due to the small number of subjects in these two groups.

TABLE 3. Peripheral blood leukocyte measures by sex

Variable	No. of cells (10^6 /ml (mean \pm SD)				Mean % leukocytes \pm SD			
	Male subjects	Female subjects	F	P	Male subjects	Female subjects	F	P
Leukocytes	6.92 \pm 2.86	7.28 \pm 2.80	1.44	>0.10	38.45 \pm 10.11	36.20 \pm 9.45	1.66	>0.10
Lymphocytes	1.55 \pm 0.52	1.58 \pm 0.53	0.83	>0.10	54.05 \pm 10.56	56.35 \pm 10.30	1.78	0.08
Granulocytes	4.25 \pm 1.91	4.54 \pm 2.01	1.45	>0.10	6.87 \pm 2.07	6.71 \pm 2.72	1.00	>0.10
Monocytes	0.51 \pm 0.23	0.49 \pm 0.21	1.20	>0.10	73.68 \pm 9.01	79.50 \pm 6.74	5.08	0.0001
T lymphocytes	1.17 \pm 0.44	1.27 \pm 0.45	0.77	>0.10	16.83 \pm 6.54	13.71 \pm 5.01	2.14	0.04
B lymphocytes	0.26 \pm 0.14	0.21 \pm 0.11	3.43	0.0009	40.16 \pm 7.09	45.64 \pm 6.71	5.85	0.0001
CD4 ⁺ lymphocytes	0.63 \pm 0.25	0.74 \pm 0.27	2.24	0.03	20.78 \pm 5.31	20.88 \pm 5.53	0.23	>0.10
CD8 ⁺ lymphocytes	0.33 \pm 0.16	0.32 \pm 0.14	0.75	>0.10	2.12 \pm 0.04	2.47 \pm 0.93	2.44	0.02
Helper-to-suppressor ratio					16.26 \pm 6.76	18.86 \pm 6.13	2.54	0.02
CD29 ⁺ lymphocytes (inducers of help)	0.27 \pm 0.15	0.30 \pm 0.14	1.17	>0.10	20.30 \pm 6.65	21.88 \pm 6.63	1.34	>0.10
CD45RA ⁺ lymphocytes (inducers of suppression)	0.33 \pm 0.17	0.35 \pm 0.17	0.61	>0.10				
CD56 ⁺ lymphocytes (NK cells)	0.08 \pm 0.10	0.06 \pm 0.06	1.56	>0.10	4.81 \pm 5.12	3.73 \pm 3.56	1.72	0.09
HLA-DR ⁺ lymphocytes (activated T cells)	0.11 \pm 0.07	0.09 \pm 0.06	1.79	0.08	6.47 \pm 4.48	5.50 \pm 3.29	1.48	>0.10

(iii) NK cell activity. There was no significant relationship between gender and mean NK cell cytotoxicity ($F = 1.08$, $P > 0.10$). When number of NK cells was controlled, these results were not altered ($F = 0.82$, $P > 0.10$).

(iv) Granulocyte activity. There were no gender-based differences in granulocyte phagocytosis ($F = 0.74$, $P > 0.10$) or killing activity at 1 h ($F = 0.15$, $P > 0.10$) or at 2 h ($F = 0.78$, $P > 0.10$) of incubation.

Race effects. (i) Cell phenotypes. The associations between race and cell numbers are presented in Table 4. WBC differed with race, being lower for African-Americans than for Latinos/Latinas ($F = 3.85$, $P < 0.0002$). African-Americans had a lower number of granulocytes than Latinos/Latinas ($F = 2.02$, $P < 0.05$) and tended to have a lower percentage of granulocytes ($F = 1.72$, $P = 0.09$). African-Americans also had a lower percentage ($F = 2.10$, $P < 0.04$) but not a lower number ($F = 1.48$, $P > 0.10$) of HLA-DR⁺ lymphocytes (activated T cells) than did Hispanics.

(ii) Mitogen-induced lymphocyte stimulation. For the proliferation assays, there were no significant effects of race on the mean lymphocyte responses to ConA ($F = 0.22$, $P > 0.10$), PHA ($F = 1.51$, $P > 0.10$), or PWM ($F = 1.27$, $P > 0.10$). However, for PWM, African-American adolescents differed from Latino/Latina youth in their dose-response curve ($F =$

9.39, $df = 2$ and 320, $P < 0.0001$) (Fig. 9). While the African-Americans seemed to show higher proliferative response at the lowest dose, the Latino/Latina adolescents showed higher proliferation at the two higher doses.

(iii) NK cell activity. There were no relationships between race and NK cell cytotoxicity ($F = 0.51$, $P > 0.10$). With number of NK cells also in the model, the relationship remained nonsignificant.

(iv) Granulocyte activity. As seen in Fig. 10, race affected granulocytic activity. Hispanic adolescents showed a higher percentage of killing than did African-American adolescents at 1 h ($F = 2.32$, $P < 0.03$) and at 2 h ($F = 1.87$, $P < 0.07$) incubation.

Substance use effects. As adolescence is a time of psychosocial as well as physical change, with frequent experimentation, including substance use, which can affect immunity, we did preliminary analyses to assess possible effects of alcohol and drug use on the immune parameters herein. Behavioral data consisted of a self-report of alcohol and drug use during the day and week preceding venipuncture and a self-report of the average number of cigarette packs the subject smoked during the past year. As substance abuse was an exclusion criterion, and as these students were either in school or actively seeking health care, the incidence of substance use was quite

TABLE 4. Peripheral leukocyte measures by race

Variable	No. of cells (10^6 /ml (mean \pm SD)				Mean % leukocytes \pm SD			
	African-American subjects	Latino/Latina subjects	F	P	African-American subjects	Latino/Latina subjects	F	P
Leukocytes	6.75 \pm 2.71	9.07 \pm 2.69	3.85	0.0002	38.23 \pm 9.80	32.22 \pm 8.28	1.61	>0.10
Lymphocytes	1.54 \pm 0.54	1.70 \pm 0.46	1.71	0.09	54.14 \pm 10.43	61.07 \pm 8.63	1.72	0.09
Granulocytes	4.12 \pm 1.84	5.78 \pm 2.01	2.02	0.05	6.90 \pm 2.44	6.18 \pm 2.39	0.48	>0.10
Monocytes	0.48 \pm 0.19	0.58 \pm 0.32	0.23	>0.10	76.46 \pm 8.46	78.03 \pm 8.16	1.10	>0.10
T lymphocytes	1.20 \pm 0.45	1.34 \pm 0.39	1.20	>0.10	15.39 \pm 5.82	14.18 \pm 6.89	1.52	>0.10
B lymphocytes	0.23 \pm 0.12	0.23 \pm 0.13	2.33	0.03	43.02 \pm 7.25	43.09 \pm 8.31	0.35	>0.10
CD4 ⁺ lymphocytes	0.67 \pm 0.26	0.75 \pm 0.29	1.01	>0.10	20.84 \pm 5.43	20.80 \pm 5.43	0.57	>0.10
CD8 ⁺ lymphocytes	0.32 \pm 0.16	0.35 \pm 0.11	1.21	>0.10	2.31 \pm 0.84	2.32 \pm 1.18	0.44	>0.10
Helper-to-suppressor ratio					17.17 \pm 6.63	19.86 \pm 5.75	1.98	>0.10
CD29 ⁺ lymphocytes (inducers of help)	0.27 \pm 0.14	0.35 \pm 0.16	0.19	>0.10	20.90 \pm 6.33	22.29 \pm 8.23	0.90	>0.10
CD45RA ⁺ lymphocytes (inducers of suppression)	0.33 \pm 0.15	0.39 \pm 0.21	0.07	>0.10	4.06 \pm 4.41	5.30 \pm 4.27	0.70	>0.10
CD56 ⁺ lymphocytes (NK cells)	0.06 \pm 0.06	0.11 \pm 0.14	1.79	0.08	5.62 \pm 3.95	7.82 \pm 3.23	2.10	0.04
HLA-DR ⁺ lymphocytes (activated T cells)	0.09 \pm 0.06	0.13 \pm 0.06	1.48	>0.10				

number of cells appears to remain stable. In our more restricted age range, we found a similar trend for an increased percentage but not an increased number of NK cells. While Comans-Bitter et al. reported no statistical findings, their observations that age may affect the percentage but not the number of NK cells are similar to ours in that numbers and/or percentages of lymphocytes can vary with age independently of each other.

Thara and colleagues (15) assessed polymorphonuclear leukocyte functioning (H_2O_2 generation, which suggests killing activity) in 58 children and adolescents and reported increased H_2O_2 production by polymorphonuclear leukocytes exposed to *S. aureus* or *Escherichia coli* with increased age (6 months to 12 years). Further, Thara et al. found that adolescents' (ages 10 to 15 years) granulocyte function (H_2O_2 production) was similar to that of adults, while children's H_2O_2 production was lower. We found that bacterial killing peaked when the subjects were 14 years old and then declined with age to values lower than those seen in the 12- to 13-year-old adolescents by age 18. The results from our study seem different in that we would predict decreased H_2O_2 production in adults compared to early adolescents, while Thara et al. found that H_2O_2 production in young adolescents and adults was quite similar. These differences may relate to the differences in study design, making the comparability of these two data sets difficult to assess (Thara and colleagues measured hydrogen peroxide generation, an indirect measure of bactericidal activity, while we measured directly the number of bacteria that were ingested and killed). Further, the data presented by Thara encompassed a much greater age range, with infants, prepubescent children, and pubescent or postpubescent adolescents, while our only subjects were postpubescent adolescents. However, despite these large methodological differences, both studies support the hypothesis that granulocyte function changes with age in young people.

Having demonstrated age-, gender-, and race-related differences in immunity during adolescence, one may speculate as to mechanisms possibly involved. Hormonal factors, especially those present during adolescence, may have influenced our results and offer venues for future investigation. For example, hormonal changes associated with growth and development may affect immunity. Indeed, peripheral blood lymphocytes have receptors for hormones such as growth hormone (GH) (20), GH releasing factor (GRF), and somatostatin (5), and some peripheral blood lymphocytes have been found to produce GH, somatostatin, and a GRF-like peptide (5). Some of the age-related differences we found might be related to GH or GRF, but it is possible that the majority of the 12- to 18-year-old subjects we studied were actively growing with similar levels of growth hormones.

The possibility that other growth-related hormonal factors account for some of the age-related differences we describe is suggested by the literature concerning the secretion of insulin-like growth factor (21), which is produced in response to secretion of GH. The secretion of insulin-like growth factor increases in both male and female adolescents, peaks higher and earlier in girls, and decreases during the latter half of adolescence to adult levels (1). This finding may help explain the age differences and suggests that age effects may be subsumed in gender findings, as girls have the most rapid growth during early puberty (before the onset of menses), while boys grow more during midpuberty (25). We undertook age by gender interaction tests to explore this and found only one significant measure (age by sex test for B-cell numbers, $P < 0.04$). Future studies comparing adolescents to same-sex fully mature adults may further delineate this issue.

Sex steroids, which also change significantly during pubertal development and continue to fluctuate in diurnal or monthly rhythms, may also have influenced our findings. Estrogen and/or progesterone may affect immunity directly or indirectly (2, 10, 23, 24, 27, 30, 32, 33, 37). Enumerative (34, 38) and functional myeloid (16, 17, 29, 36) and lymphoid (7, 28, 37, 39, 42) measures have been reported to be affected by sex steroids.

Enumerative immune measures may be affected by sex steroids. Casson and colleagues (6) reported that the percentage of CD4⁺ cells was decreased, while that of NK cells was increased, when postmenopausal women were treated with dehydroepiandrosterone. Kless and coworkers (22) reported lower percentages of CD4⁺ cells in untreated males with hypogonadism compared to those in normal healthy men and those in subjects with treated hypogonadism, which resulted in normal testosterone levels. Our finding of lower numbers of CD4⁺ cells in males is consistent with Casson and coworkers' (6) findings suggesting that circulating androgens (in postmenopausal women) are associated with decreased numbers of CD4⁺ cells.

Concerning sex steroid effects on mitogen stimulation, Yron and colleagues (42) found that neither 17 beta-estradiol nor progesterone altered the response to ConA. We found no gender differences in T- or B-cell response to mitogen stimulation (PHA, PWM, or ConA). Therefore, our findings are consistent with those of Yron et al. and suggest that response to mitogen stimulation is not significantly affected by gender differences in gonadal hormones.

Contradictory findings for effects of sex steroids on NK activity have been reported. For example, Sorachi and coworkers (37) reported that 17 beta-estradiol (E2) enhances NK activity, while progesterone and testosterone do not. Liu and Hansen (26) reported that NK cytotoxic activity was inhibited by progesterone. Mandler and coworkers (27) reported that NK cell depolarization is affected by progesterone but not by estrogen. Contrarily, Callewaert and coworkers (4) reported that NK cell activity was not affected by high concentrations in vitro of testosterone, progesterone, or estradiol. We did not find any gender (or age \times sex)-related differences in NK cell activity, and therefore our findings are more in keeping with the implications of the results reported by Callewaert and colleagues. However, we did not control for stage of menstrual cycle, which at peaks of estrogen or progesterone might have yielded very different results.

In addition to age and gender effects, we found significant racial differences in some enumerative measures and in granulocyte function (African-Americans compared to Latino/Latina youth). Tollerud and coworkers also found racial differences in enumerative measures (percentage of HLA-DR⁺ (Caucasians versus blacks). Thara and colleagues (15) did not indicate their subjects' race, but presumptively their sample was all Japanese, thereby precluding any investigation of racial differences. The biological basis of racial differences remains to be explored. However, this data suggests the existence of racial differences in immunity which must be addressed whenever immune-related disease processes are investigated.

The potential for selection bias in this data must be addressed. The subjects in the present study were recruited from an inner-city high school and an inner-city adolescent general medical clinic. Students were members of randomly selected 10th grade English classes, and greater than 90% of the students in each targeted class agreed to participate. Similarly, in the adolescent clinic, consecutive patients were approached for participation in the study, and greater than 80% of these adolescents agreed to participate. Results from studies with high school dropouts or children with academic problems requiring

special education services might be dissimilar to those of the present study.

Concerning the physical well-being of the subjects in the present study, all were categorized as healthy by physicians, based on both history and physical examination. Since these subjects were part of a larger study on behavioral and biological AIDS risk factors, we also collected a large, intimate behavioral and psychological data set on the same day as venipuncture. The subjects had no reason to misrepresent their health, and they were asked much more personal information than when they had most recently been ill. The time frame for recall of intercurrent health problems was short (2 weeks) and thus should not have represented a problem in recall. The random selection of the subjects, the physical assessment by physicians, the collection of intimate psychobehavioral data and venipuncture on the same day, as well as the immediate processing of the blood for each of the multiple immune assays were important factors in assuring the quality of this normative data.

The effects of demographic factors on a wide range of immunological variables demonstrate the importance of having normative data representative of particular patient populations. Even though our subjects were randomly sampled from the same general population, there were marked immunological differences in subgroups defined by age, gender, and race. If the stability of these factors over time is addressed in these types of studies, researchers will have an even clearer picture of the normative values of immunological functioning in adolescents.

REFERENCES

- Allen, D. B., A. J. Johanson, and R. M. Blizzard. 1996. Growth hormone treatment. In F. Lifshitz (ed.), *Pediatric endocrinology*. Marcel Dekker, Inc., New York, N.Y.
- Athreya, B. H., J. Fletcher, J. Zullian, D. B. Weiner, and W. V. Williams. 1993. Subset specific effects of sex hormones and pituitary gonadotropins on human lymphocyte proliferation in vitro. *Clin. Immunol. Immunopathol.* 66:210-211.
- Birmaher, B., B. S. Rabin, M. R. Garcia, U. Jain, T. L. Whiteside, D. E. Williamson, M. al-Shabbaz, R. C. Nelson, R. E. Dahl, and N. D. Ryan. 1994. Cellular immunity in depressed, conduct disorder, and normal adolescents: role of adverse life events. *J. Am. Acad. Child Adolesc. Psychiatry* 33:671-678.
- Calleraert, D. M., V. K. Moudgil, G. Radcliff, and R. W. Wooten. 1991. Hormone specific regulation of natural killer cells by cortisol. *Direct modulation of the cytotoxic function of cloned human NK cells without an effect on cellular proliferation*. *FEBS Lett.* 289:108-110.
- Campbell, R. M., and C. G. Scanes. 1995. Endocrine peptides 'moonlighting' as immune modulators: roles for somatostatin and GHRH-releasing factor. *J. Endocrinol.* 147:383-396.
- Carlson, P. R., L. C. Faguna, F. B. Sientz, A. B. Staughn, R. N. Andersen, G. E. Abraham, and J. E. Buster. 1993. Replacement of dehydroepiandrosterone enhances T-lymphocyte insulin binding in postmenopausal women. *Fertil. Steril.* 63:1027-1031.
- Clerici, M., L. DePalma, S. Rotondo, R. Baker, and G. Shearer. 1993. Analysis of T helper and antigen-presenting cell function in cord blood and peripheral blood leukocytes from healthy children of different ages. *J. Clin. Invest.* 91:2629-2636.
- Comans-Bitter, W. M., R. deGroot, R. van de Beem, H. J. Neijens, W. C. Hop, K. Greenfield, H. Hoofkaas, and J. J. M. van Dongen. 1997. Immunophenotyping of blood lymphocytes in childhood. *J. Pediatr.* 130:388-393.
- Dryden, R. A., and B. A. Aronow. 1992. Natural regulators of T-cell lymphokine production in vivo. *J. Immunother.* 12:174-179.
- Ergulalp, M., and J. Farrard. 1994. Effect of 17 beta-estradiol on immunoglobulin secretion by human tonsillar lymphocytes in vitro. *J. Steroid Biochem. Mol. Biol.* 48:171-177.
- Gemas-Engels, V., A. B. Kay, A. Bush, and C. J. Corrigan. 1994. Activated peripheral blood CD4 and CD8 T-lymphocytes in child asthma: correlation with eosinophilia and disease severity. *Pediatr. Allergy Immunol.* 9:170-177.
- Georgescu, R., and S. E. Keller. 1987. Overnight storage at 1°C does not affect NK cytotoxicity. *J. Immunol. Methods* 109:151.
- Georgescu, R., and S. E. Keller. 1987. Decreasing the spontaneous release in NK cell assays. *J. Immunol. Methods* 103:143.
- Gillis, S., M. M. Ferm, et al. 1978. T cell growth factor: parameters of production and a quantitative microassay for activity. *J. Immunol.* 120:2027-2032.
- Ikura, T., K. Yamawaki, T. Fujitani, K. Kitamura, M. Sakurai, and H. Kameya. 1994. A flow cytometric method for measurement of hydrogen peroxide generation by pediatric polymorphonuclear leukocytes stimulated by *Staphylococcus aureus* and *Escherichia coli*. *Acta Paediatr. Jpn.* 34:244-249.
- Ito, J., T. Hayashi, K. Yamada, M. Kuroya, M. Naito, and A. Iguchi. 1995. Physiological concentration of estradiol inhibits polymorphonuclear leukocyte chemotaxis via a receptor mediated system. *Life Sci.* 56:2247-2253.
- Jaansson, G. 1991. Oestrogen-induced enhancement of myeloperoxidase activity in human polymorphonuclear leukocytes—a possible cause of oxidative stress in inflammatory cells. *Free Radical Res. Commun.* 14:199-208.
- Jenkins, C. D., B. E. Kregen, R. M. Ross, and M. Hurst. 1980. Use of monthly health review to ascertain illness and injuries. *Am. J. Public Health* 70:82-84.
- Keller, S. E., S. J. Scheller, J. Sherman, M. Cameron, H. Smith, Jr., and M. Stein. 1981. Comparison of a simplified whole blood and isolated lymphocyte stimulation technique. *Immunol. Commun.* 10:417-431.
- Kelly, K. W. 1989. Concomitant—growth hormone, lymphocytes and macrophages. *Biochem. Pharmacol.* 38:705-713.
- Kelly, K. W., S. Arkins, and M. Y. Li. 1992. Growth hormone, prolactin, and insulin-like growth factors: new jobs for old players. *Brain Behav. Immun.* 6:317-326.
- Kies, W., L. L. Lin, and N. R. Hall. 1991. Lymphocyte subset distribution and natural killer cell activity in men with idiopathic hypogonadotropic hypogonadism. *Acta Endocrinol.* 124:399-404.
- Kisnado, P. W., K. L. Medina, and G. Smithson. 1994. Sex hormones as negative regulators of lymphopoiesis. *Immunol. Rev.* 137:119-134.
- Li, Z. C., V. A. Davis, and P. M. Brooks. 1993. Effects of gonadal steroids in the production of IL-1 and IL-6 by blood mononuclear cells in vitro. *Clin. Exp. Rheumatol.* 11:157-162.
- Lifshitz, F., and C. D. Cervantes. 1996. Short stature. In F. Lifshitz (ed.), *Pediatric endocrinology*. Marcel Dekker, Inc., New York, N.Y.
- Li, W. J., and P. J. Hansen. 1993. Effects of the progesterone-induced serpin-like proteins of the sheep endometrium on natural-killer cell activity in sheep and mice. *Biol. Reprod.* 49:1008-1014.
- Mandler, R. N., L. C. Scanes, M. D. Donsawski, and A. D. Bankhurst. 1993. Progesterone but not estrogen depolarizes natural killer cells. *Nat. Immun.* 12:128-135.
- Manoyada, I. T., R. S. Pandey, V. Makinda, M. Briscoe, and R. T. Varma. 1992. Effect of 17 beta-estradiol on lymphocyte subpopulations, delayed cutaneous hypersensitivity responses and mixed lymphocyte reactions in postmenopausal women. *Maturitas* 14:201-210.
- Miyagi, M., H. Aoyama, M. Morishita, and Y. Iwamoto. 1992. Effects of sex hormones on chemotaxis of human peripheral polymorphonuclear leukocytes and monocytes. *J. Periodontol.* 63:28-32.
- Moldofsky, E. 1994. Central nervous system and peripheral immune function and the sleep-wake system. *J. Psychiatry Neurosci.* 19:368-374.
- Nova, E., M. I. Garcia, and J. Laverne. 1993. Non-specific immunity in Down syndrome: a study of chemotaxis, phagocytosis, oxidative metabolism, and cell surface marker expression of polymorphonuclear cells. *Am. J. Med. Genet.* 46:384-391.
- Oner, P., S. Bekpazar, F. Cinar, and A. Argun. 1994. Relationship of type endogenous sex steroid hormones to leukocyte arylsulphatase A activities in pre and post menopausal healthy women. *Horm. Metab. Res.* 26:301-304.
- Pacifici, R., J. L. Vassallo, L. Rifas, and R. E. Kimble. 1993. Monocytic secretion of interleukin-1 receptor antagonist in normal and osteoporotic women: effects of menopause and estrogen/progesterone therapy. *J. Clin. Endocrinol. Metab.* 77:1135-1141.
- Pierjani, M. P., M. G. Giudizi, R. Biagiotti, L. Beloni, L. Giannarini, S. Samponaro, P. Farrochi, R. Mastelli, F. Annunziati, and C. Livi, et al. 1995. Progesterone favors the development of human T helper cells producing Th2-type cytokines and promotes both IL-4 production and membrane CD30 expression in establish Th1 cell clone. *J. Immunol.* 155:128-133.
- Rabin, R. L., M. Roederer, Y. Makhoul, A. Petru, L. A. Herzenberg, and L. A. Herzenberg. 1995. Altered representation of naive and memory CD8 T cell subsets in HIV-infected children. *J. Clin. Invest.* 95:2034-2040.
- Shibuya, T., K. Inuchi, A. Kuroiwa, H. Harada, A. Kunitake, and K. Shirakawa. 1991. Study on non-specific immunity in pregnant women. II. Effects of hormone on chemiluminescence response of peripheral blood phagocytes. *Am. J. Reprod. Immunol.* 24:76-81.
- Sorachi, K., S. Kumagai, M. Sugita, J. Yodol, and H. Imura. 1993. Enhancing effects of 17 beta-estradiol on human NK cell activity. *Immunol. Lett.* 34:31-35.
- Sridama, V., S. Limpooguanurak, S. Sripongayawan, and S. Youngpraporn. 1992. Decreased suppressor T-lymphocytes in women who received progesterone injections. *J. Med. Assoc. Thai.* 75:479-482.
- Suzuki, T., N. Suzuki, R. A. Daynes, and E. G. Engleman. 1991. Dehydro-

- epiandrosterone enhances IL-2 production and cytotoxic effector function of human T cells. *Clin. Immunol. Immunopathol.* 61:202-211.
40. Tollerud, D. J., S. T. Mestad, L. M. Brown, J. W. Clark, W. A. Battister, D. L. Mann, C. Y. Neufeld, L. Paulsen-Trost, and R. N. Roover. 1990. T-cell subsets in healthy teenagers: transition to the adult phenotype. *Clin. Immunol. Immunopathol.* 56:88-96.
41. Weir, D. M. (ed.). 1978. *Handbook of experimental immunology*, 3rd ed. Blackwell Scientific Publications Ltd., Oxford, England.
42. Yron, I., A. Langer, T. Weinstein, E. Sahar, Y. Lidor, Y. Pardo, I. Katz, L. Shohat, Y. Kaleshman, J. Ovadia, et al. 1997. Effects of sex hormones in human T cell activation by concanavalin A. *Nat. Immun. Cell Growth Regul.* 10:32-44.

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Characterization of antibodies submitted to the B cell section of the 8th Human Leukocyte Differentiation Antigens Workshop by flow cytometry and immunohistochemistry

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Abstract

The aim of this study was to characterize the reactivity of monoclonal antibodies (mAbs) that had been submitted to the HLDA8 Workshop. The lineage specificity of target molecules was tested by analyzing their expression patterns on blood cells, leukocytes, and lymphocyte subsets. The expression of target molecules during B cell development, ranging from early precursors to plasma cells, was analyzed using a large panel of B cell lines. Our results have permitted us to characterize the expression of 10 new CD molecules: CD316 (HM1.24, BST2), CD268 (BAFF-R, TNFRSF13C), CD269 (BCMA, TNFRF17), CD267 (TACI, TNFRSF13B), CD275 (ICOSL, B7H2), CD254 (TRANCE, TNFSF11), CD252 (OX40, TNFSF4), CD315 (CD9-P), CD316 (EWI-2, PGRL), and CD307 (IRTA-2 or FeRH5). Three of these new CDs, CD267, CD269, and CD307 presented a B cell-restricted expression pattern. MAb against these novel cell-surface molecules may offer new tools for research, diagnosis, and therapy.
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Keywords: Leukocytes; B cells; Cell-surface molecules; Monoclonal antibodies; CD

1. Introduction

Many events regulating the development, activation, and effector functions of lymphocytes are orchestrated by a complex series of cell-surface molecules. Some of these molecules are expressed by several lymphocyte subsets and are differentially expressed during lymphocyte development and activation [1]. The production of monoclonal antibodies (mAbs) capable of identifying single antigens has led to the discovery of a large number of leukocyte cell-surface molecules with novel activities. Human leukocyte differentiation antigens (HLDA)

Workshops have proven instrumental in the identification and characterization of a large number of molecules that populate the surface of hematopoietic cells [2,3]. At the last of these Workshops (HLDA8), held in Adelaide, Australia in December 2004, 95 new clusters of differentiation (CD) antigens were allocated, bringing the total number to 339 CDs [4]. MAb are essential tools in determining the expression, function, and molecular structure of these molecules, and greatly improved our understanding of the cellular and molecular interaction involved in immune responses. Most of the mAbs submitted to the HLDA8 Workshop were raised against recombinant proteins or transfected cells. However, the expression patterns of these cell-surface molecules are often poorly understood even for molecules well characterized both at the structural and functional levels.

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Here we characterize the expression of target molecules recognized by those mAbs submitted to the B cell Section of the HLDA8 Workshop.

2. Materials and methods

2.1. Antibodies

Sixty-six monoclonal antibodies (mAbs) submitted to the B Cell Section of the HLDA8 Workshop were studied. Only those mAbs that recognized antigens expressed on the cell-surface were included in this study (Table 1). Moreover, any of the submitted mAb that recognized known molecules, but which could not be validated by the B Cell Section using transfected cells were excluded. Study mAbs belonged to one of two categories: those directed against potential new CD8 (18 mAbs) or those against unknown molecules (14 mAbs). Membrane bound mouse mAbs were detected using biotinylated anti-mouse light κ chain (Becton–Dickinson, Immunocytometry Systems, San Jose, CA), followed by Streptavidin-PE (Becton–Dickinson) for flow cytometry studies. A FITC-conjugated anti-rat IgG antiserum (Caltag Lab-

oratories, Burlingame, CA) was used for the detection of rat mAbs.

The following mAbs were obtained from Becton–Dickinson: CD16-Cy-Chrome, CD69-FITC, CD80-PE, and CD44-FITC.

2.2. Cell lines

The 19 haemopoietic cell lines analyzed were obtained from the American Type Culture Collection (Manassas, VA), with the exception of BEN and I38-E95, which were produced by transforming peripheral blood leukocytes with Epstein–Barr virus (Table 2). Cell lines were cultured in complete medium (RPMI 1640 supplemented with 10% fetal calf serum (FCS), antibiotics, and 10 mmol/L glutamine) (Gibco-BRL) and then split twice weekly at a ratio allowing maintenance of a cell concentration below 10^6 cells/mL.

2.3. Leukocytes

Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll density gradient centrifugation from buffy coats drawn from healthy donors. Tonsils were obtained

Table 1
B cells section mAbs

Code	Workshop No.	Submitter	Clone name	Isotype	Species
B001	80109	Imanishi	37.20	IgG	Rat
B002	80110	Imanishi	38.12	IgG	Rat
B003	80114	Koishi-hara	HM1.24	IgG2a	Mouse
B004	80645	Koishi-hara	RS38	IgG2a	Mouse
B005	80646	Koishi-hara	K5C1	IgG2a	Mouse
B006	80148	Nikolova-Stoimenova	SC3	IgM	Mouse
B007	80309	Nozawa	FH25	IgG1	Mouse
B008	80310	Nozawa	FB21	IgM	Mouse
B009	80323	Werner Maga	HIL-131	IgG1	Mouse
B010	80395	Shen	HI220	IgG3	Mouse
B011	80397	Shen	HI301	IgG1	Mouse
B012	80398	Shen	HI305	IgM	Mouse
B013	80399	Shen	HI306	IgG2a	Mouse
B014	80400	Shen	HI307	IgG1	Mouse
B015	80443	Shen	HI302	IgG1	Mouse
B016	80445	Rubinstein	1F11	IgG1	Mouse
B017	80446	Rubinstein	KA12	IgG2a	Mouse
B019	80460	Mortari	70513	IgG1	Mouse
B021	80155	Macardie	FMC68	IgM	Mouse
B022	80156	Macardie	FMC76	IgM	Mouse
B023	80278	Voland	E63-761	IgG2a	Mouse
B026	80577	Murone	Vioky-1	IgG1	Rat
B028	80481	Sin	FMU-TAC12	IgG1	Mouse
B051	80504	Sin	FMU-BCMA1	IgG1	Mouse
B053	80558	Zhang	51CD134L-2(4C12)	IgG2b	Mouse
B057	80585	Zhang	51CD137L-1F1	IgG1	Mouse
B058	—	Hemler	9A5	IgM	Mouse
B059	80690	Nagata	F56	IgG1	Mouse
B060	80689	Nagata	F29	IgG2a	Mouse
B061	80688	Nagata	F119	IgG2b	Mouse
B062	80683	Mackay	1A1	IgG1	Rat
B063	80684	Mackay	11C1	IgG1	Mouse

Table 2
Cell lines

<i>B cell lines</i>	
REH	Pro-B cell leukemia
JM1	Pre-B cell leukemia
RAMOS	B cell lymphoma
RAJI	Burkitt lymphoma
DAUDI	Burkitt lymphoma
NAMALWA	Burkitt lymphoma
REC1	Mantle cell lymphoma
JVM-2	Mantle cell lymphoma
GRANTA-519	Mantle cell lymphoma
NCEB	Mantle cell lymphoma
CESS	EBV-transformed B cells
BEN	EBV-transformed B cells
138-E95	EBV-transformed B cells
RPMI 8226	Multiple myeloma
<i>Non-B cell lines</i>	
JURKAT	T cell leukemia
HSB2	T cell leukemia
YT	T/NK cell leukemia
HL-60	Mycloid leukemia
K-562	Mycloid leukemia
U-937	Mycloid leukemia

from patients undergoing tonsillectomy for breathing disorders. Lymphocyte suspensions were prepared by teasing the tissue in RPMI 1640 medium (Gibco-BRL, Gaithersburg, MD) with 10% FCS (Gibco-BRL) and were passed through silk screen to remove any connective tissue. Lymphocytes were then isolated by Ficoll density gradient centrifugation (1800 rpm for 25 min). B cells were obtained following treatment with anti-CD3 mAb (90×10^6 cells/ $10 \mu\text{g}$ anti-CD3) plus rabbit serum as a source of complement in RPMI 10% FCS. All tissues were stored in RPMI 1640 (Gibco-BRL) medium at 4°C until further processing. Typically, the tissues were processed within 1–3 h following surgical removal. Granulocytes were identified by their forward- and side-scatter characteristics, as well as by CD16 expression [5].

2.4. *In vitro* cell stimulation

PBMCs were activated with 1% (v/v) phytohemagglutinin (PHA) (Murex Biotech Limited, Dartford, England) for 72 h at concentrations of 2×10^6 cells/mL in RPMI 10% FCS. B lymphocytes from tonsils were activated with 1% (v/v) Prot A-Sepharose Beads (Bio-Rad, Hercules, CA) for 72 h at concentrations of 2×10^6 cells/mL in RPMI 10% FCS.

2.5. Monocyte-derived dendritic cells

Aliquots of 50×10^6 peripheral blood mononuclear cells were incubated for 2 h at 37°C and 5% CO_2 in a 25cm^2 flask (Nunc, Denmark) in RPMI 1640 medium (Gibco-BRL). After washing, adherent monocytes were cultured in the presence of 500 U/mL of IL-4

(Sigma) and 1000 U/mL of GM-CSF (Leucomax, Novartis, Switzerland) in RPMI 1640 medium with 10% FCS (Gibco-BRL) at 37°C in a humidified atmosphere of 5% CO_2 . Purity of immature DC was typically $>95\%$, as determined by flow cytometry using mAb CD80-PE.

2.6. Natural killer cells

Peripheral blood lymphocytes (PBLs) were isolated by Ficoll (Sigma) density gradient centrifugation from buffy coats drawn from healthy donors. Mitomycin C (0.5 mg/mL , Sigma) pre-treated Daudi cells were mixed with PBL at a ratio 4:1 (PBL/Daudi). These cells were incubated for 6–7 days in RPMI 10% FCS at 5% CO_2 humidified atmosphere. Homogeneous populations of NK cells were obtained by subsequent treatment with anti-CD3 mAb (90×10^6 cells/ $10 \mu\text{g}$ anti-CD3) plus rabbit serum as a source of complement. Purity of NK cells was determined by CD56 expression.

2.7. Red blood cells and platelets

Human blood cells were collected in Vacutainer tubes containing sodium citrate 0.129 M (Becton-Dickinson). Blood was centrifuged at $400g$ for 10 min at room temperature to isolate platelet-rich-plasma (PRP). The PRP was then resuspended with equal volumes of phosphate buffer solution (PBS). Platelets were further isolated by centrifugation of PRP at $2000g$ for 6 min, washed with PBS and centrifuged again at $2000g$ for 6 min. Platelets were then re-suspended with PBS containing 0.5% FCS.

Human blood cells were washed twice with PBS (2200 rpm for 5 min) at room temperature to isolate red blood cells.

2.8. CD34⁺-cells

rhG-CSF (Amgen, Thousand Oaks, CA) was administered to donors at a dose of $10 \mu\text{g/kg}$ per 12 h via subcutaneous injections for up to 7 days until harvesting. CD34⁺ cells were positively selected using immunomagnetic via Isolex 300i (Baxter, Munich, Germany) and ClinMACS (Miltenyi 1 Biotec, Bergisch Gladbach, Germany). Purity of the bone-marrow precursor cells was determined by CD34 expression (median = 98.61% storage 93.1–99.74%).

2.9. Immunofluorescence analysis

Cells were stained using biotinylated anti-mouse light κ chain (Becton-Dickinson, Immunocytometry Systems, San Jose, CA) followed by Streptavidin-PE (Becton-Dickinson). Fluorescence was analyzed using a FAC-SCalibur (Becton-Dickinson) flow cytometer equipped with CellQuest software. Fluorescence intensity was plotted on a log scale. A minimum of 5000 cells was

Human cells and tissues used in this study were obtained in compliance with protocols approved by the Ethics Committee of the Hospital Clinic of Barcelona (Barcelona, Spain) and according to the principles of the Declaration of Helsinki.

3. Results

3.1. Characterization of monoclonal antibodies that recognize unknown B cell associated molecules

37.20 (B001). This mAb recognized a cell-surface molecule expressed on mature B cells (Tables 3 and 4). The expression of this cell-surface molecule was increased after activation (Table 3). In contrast, its expression on peripheral blood T cells was very low (Table 5). During B cell development, as reflected by its presence on the B cells and B cell lines analyzed, we observed that its expression began in pre-B cells and reached peak levels

Code	Clone name	B Cells	Activated B cells (Prot A)	REH	JM1	DAUDI	RAMOS	RAJI	NAMALWA
B001	37.201	++++	++++ ↑	-	+++	++++	++++	++++	-
B002	38.12	++++	ND	++++	++++	++++	++++	++++	+++
B003	HM124	++++	++++ ↑	+++	++	++++	++++	++++	++++
B004	RS38	+++	++++	+	-	++++	++++	+++	++++
B005	K5C1	+++	++++	++	+	++++	++++	++++	++++
B006	SC3	-	+++	-	-	-	-	+	-
B007	FH25	+++	++++	++++	-	+++	-	++++	-
B008	FB21	++++	++++ ↑	++++	++++	++++	++++	++++	++++
B009	HTL-131	++	+++	++++	++++	++++	++++	++++	++++
B010	HI220	++++	ND	++++	++++	++++	++++	++++	++++
B011	HI301	++++	ND	++++	++++	++++	++++	++++	++++
B012	HI305	-	+++	-	-	-	-	-	+
B013	HI306	++++	++++ ↑	++++	++++	++++	++++	++++	++++
B014	HI307	++++	ND	++++	++++	++++	++++	++++	++++
B015	HI302	++++	ND	++++	++++	++++	++++	++++	++++
B016	1F11	-	-	-	-	-	-	-	+++
B017	8A12	++++	++++ ↑	++++	++	++++	++++	++++	++++
B019	70513	-	ND	-	-	-	+	++	-
B021	FMC68	++++ bi	ND	ND	-	++++	++++	++++	++++
B022	FMC76	++++ bi	ND	ND	-	++++	-	++++	-
B023	E63-761	ND	ND	++++	++++	++++	++++	++++	++++
B026	Vicky-1	ND	-	-	-	-	-	-	-
B028	FMU-TAC12	-	ND	ND	-	++++	-	+++	-
B051	FMU-BCMA1	+	-	ND	+	+++	+++	++	-
B053	SICD134L-2(4C12)	++++	++++ ↑	++++	+	++++	++++	++++	++++
B057	SICD137L-1F1	-	+	-	+	-	-	+	-
B058	9A5	-	+	++++	++++	++++	++++	+++	++++
B059	F36	++	++++	-	-	-	-	-	++++
B060	F25	+	++++	-	-	-	-	-	+++
B061	F119	+	++++	-	-	-	-	-	+++
B062	1A1	-	-	-	+	-	+	-	-
B063	11C1	++++	++++ ↑	++++	+	++++	++++	++++	++++

The cells were incubated with the different mAbs. Then cells were washed and incubated with biotinylated anti-mouse light k chain followed by streptavidin-PE. A FITC-conjugated anti-rat IgG antiserum was used with rat mAbs. Results represent the percentage of positive cells: -, <20% of positive cells; +, 20-40% of positive cells; ++, 40-60% of positive cells; +++, 60-80% of positive cells; +++++, 80-100% of positive cells; ND: not done; ↑: increment of the molecule expression with activation; bi: bimodal.

Table 4
Reactivity of the mAbs with B cell lines

Code	Clone name	CESS	BEN	I38-E95	RPMI 8226	GRANTA	JVM-2	NCEB	REC
B001	37.20	++++	++++	+++	–	+	++	++++	+++
B002	38.12	++++	++++	++++	–	++++	++++	++++	–
B003	HM1.24	++++	++++	++++	++++	++++	++++	++++	++++
B004	RS38	+++	+++	++++	++++	++++	++	+++	++++
B005	K5C1	++++	++++	++++	++++	+++	++++	++++	++++
B006	SC3	–	–	++	+	++	++	++++	+++
B007	FH25	+++	+++	++++	–	++++	++++	++++	+
B008	FB21	++++	++++	++++	–	++++	++++	++++	++++
B009	HIL-131	++++	++++	++++	++++	++++	++++	++++	++++
B010	M220	++++	++++	++++	–	++++	++++	++++	–
B011	HI301	++++	++++	++++	+	++++	++++	++++	–
B012	HI305	+	–	+++	+++	+	++	++++	+
B013	HI306	++++	++++	++++	+	++++	++++	++++	–
B014	HI307	++++	++++	++++	–	–	++++	++++	–
B015	HI302	++++	++++	++++	–	++++	++++	++++	–
B016	IF11	+	–	–	–	–	–	–	–
B017	8A12	++++	++++	++++	++++	++++	++++	++++	++++
B019	7Q513	++++	–	–	+	–	–	–	++++
B021	FMC68	++++	++++	++++	–	+	+	+++	++++
B022	FMC76	++++	++++	++++	–	++++	++++	++++	–
B023	E63-761	++++	++++	++++	–	++++	++++	++++	–
B026	Vicky-1	++	–	–	–	–	–	–	–
B028	FMU-TAC12	++	+	++	++	–	+	+	+
B051	FMU-BCMA1	++++	++	+++	++++	++	+++	+++	++++
B053	SICD134L-2(4C12)	++++	+++	++++	+++	++++	++++	++++	++++
B057	SICD137L-1F1	++	–	–	–	++	–	–	–
B058	9A5	++++	++++	++	++++	++++	++	–	+++
B059	F56	++	–	++++	–	++++	+	+	++++
B060	F25	++	–	++++	–	+++	+	+	++++
B061	F119	++	–	++++	–	+++	+	–	++++
B062	1A1	++++	+++	+++	–	–	++++	–	++++
B063	11C1	++++	++++	++++	–	++++	++++	++++	++++

The cells were incubated with the different mAbs. Then cells were washed and incubated with biotinylated anti-mouse light κ chain followed by streptavidin-PE. A FITC-conjugated anti-rat IgG antiserum was used with rat mAbs. Results represent the percentage of positive cells: –, <20% of positive cells; +, 20–40% of positive cells; ++, 40–60% of positive cells; +++, 60–80% of positive cells; +++++, 80–100% of positive cells.

on activated B cells (Table 3). Strong expression in the mantle zone of tonsil sections was observed (Table 7).

SC3 (B006). The cell-surface molecule recognized by this mAb was expressed on activated but not on resting B cells (Table 3). All analyzed B cells were negative except for low levels found on mantle zone B cell and plasma cell lines (Tables 3 and 4). Expression was not restricted to B cells since high levels were detected on granulocytes and dendritic cells, and to a lesser extent, on red blood cells and platelets (Table 6).

FH25 (B007). The molecule recognized by this mAb was present on mature and activated B cells (Table 3). Interestingly, high levels could be detected on the pro-B cell line REH (Table 3). Its expression was not B cell restricted since it was also highly expressed on resting and activated T cells as well as monocytes. It was weakly expressed on NK cells and granulocytes (Tables 5 and 6).

FB21 (B008). This mAb reacted strongly with B and T cells, granulocytes, monocytes, and NK cells, less with dendritic and red blood cells (Tables 3, 5, and 6). Its reac-

tivity increased with B cell activation (Table 3). In fact, during B cell development, reactivity crossed from pro-B cells to activated B cells (Table 3). Strong staining of the mantle zone was observed (Table 7).

HI305 (B012). This mAb reacted with activated B and T cells (Tables 3 and 5). No staining was detected with immature B cells (Table 3) and strongest reactivity was found with granulocytes. Lower reactivity was detected with monocytes and platelets (Table 6).

38.12 (B002), HI220 (B010), HI301 (B011), HI306 (B013), HI307 (B014), HI302 (B015), and E63-761 (B023). Seven of the submitted mAbs were highly reactive with B cells and other APC's (Table 3). Since reactivity patterns were reminiscent of mAbs directed against MHC class II molecules, we tested their reactivity on the Raji cell line, and as well as a Raji mutant expressing low levels of these molecules. All of these mAbs exhibited greatly reduced reactivity with the mutant as compared to the wild type Raji cell line (Table 8). To further demonstrate their specificity, we performed immunoprecipitations from Raji lysates. Our results showed that all of these mAbs

Table 5
Reactivity of the mAbs with CD34⁺ precursors, T cells, and T cell lines

Code	Clone name	CD34 ⁺ precursors	T cells	Activated T cells (PHA)	JURKAT	HSB2	NK	YT
B001	37.20	—	++	+	—	—	—	—
B002	38.12	—	—	ND	—	—	ND	++++
B003	HM1.24	++++	++++		+		++++	++++
B004	RS38	++++	++++	++++	—	++++	++	++++
B005	K5C1	+++	+++	++++	—	++++	+++	++++
B006	SC3	—	—	—	—	—	—	—
B007	FH25	—	++++	++++ ↑	++++	+	+	++++
B008	FB21	—	++++	++++	—	—	++++	++++
B009	HIL-131	—	+	+	—	++	—	—
B010	HI220	—	—	ND	—	—	ND	++++
B011	HI301	—	—	ND	—	—	ND	++++
B012	HI305	—	—	++	—	+	—	—
B013	HI306	—	—	+++	—	—	—	++++
B014	HI307	—	—	ND	—	—	ND	++++
B015	HI302	—	—	ND	—	—	ND	++++
B016	1F11	—	—	—	—	—	—	—
B017	8A12	—	+++	+++	++++	++++	+++	++++
B019	70513	—	—	—	—	—	ND	—
B021	FMOC68	—	—	ND	—	+	ND	—
B022	FMC76	—	—	ND	—	+	ND	—
B023	E63-761	—	—	ND	—	—	ND	++++
B026	Vicky-1	—	—	—	—	—	—	—
B028	FMU-TAC12	—	—	ND	—	+++	ND	—
B031	FMU-BCMA1	—	—	ND	—	+	ND	—
B053	SIQD134L-2(4C12)	++	—	—	—	+	—	—
B057	SIQD137L-1F1	—	—	—	++++	++	—	+
B058	9A5	—	+++	+	++++	++++	—	++++
B059	F56	—	—	—	—	—	—	—
B060	P25	—	—	—	—	—	—	—
B061	F119	—	—	—	—	—	—	—
B062	1A1	—	—	—	—	—	—	—
B063	11G1	—	+	+	+	—	—	—

The cells were incubated with the different mAbs. Then cells were washed and incubated with biotinylated anti-mouse light κ chain followed by streptavidin-PE. A FITC-conjugated anti-rat IgG antiserum was used with rat mAbs. Results represent the percentage of positive cells: —, <20% of positive cells; + 20–40% of positive cells; ++, 40–60% of positive cells; +++, 60–80% of positive cells; +++++, 80–100% of positive cells; ND; not done; ↑: increment of molecule expression with activation.

precipitated two bands of 35 and 24 kDa, characteristic of MHC class II molecules (data not shown).

3.2. Characterization of monoclonal antibodies that recognize known molecules with unknown CD's

HM1.24 (B003), RS38 (B004), and K5C1 (B005). These three mAbs recognized BST2 (bone marrow stromal antigen 2). This molecule was highly expressed during B cell development, from pro-B precursors to plasma cells and its expression increased with activation (Tables 3 and 4). CD34⁺ bone-marrow cells were also positive (Table 5). In addition, BST-2 was highly expressed on T cells, monocytes, NK cells and dendritic cells (Tables 5 and 6). All B cell lines tested were positive, except for the pro-B cell line JM1 (Table 3). Lymphoid follicles and non-hematopoietic cells were heavily stained with these antibodies (Table 7).

HIL-131 (B009). This mAb recognized a molecule expressed by APCs with strongest expression occurring

on dendritic cells (Tables 3 and 6). Expression was also observed on peripheral blood T cells and the T cell line HSB2 (Table 5). During B cell development, it was highly expressed on pro-B, pre-B cells, and immature cells. While decreasing with maturation, expression increased following activation (Tables 3 and 4). HIL-131 mAb stained scattered cells in the interfollicular zone in tonsil sections (Table 7).

1F11 (B016). This mAb recognizes CD9P-1, also known as EWI-F. All B cells tested were consistently negative with the exception of the Burkitt lymphoma cell line Namalwa (Tables 3 and 4). Monocytes were negative but some myeloid cell lines such as HL-60, U-937, and K-562 were highly positive (Table 6). Positive staining with 1F11 mAb of non-lymphoid cells was observed in tonsils while strongest reactivity occurred with crypt epithelial cells (Table 7).

8A12 (B017) and 9A5 (B058). These mAbs are from two different laboratories and recognized EWI-2, also known as PGRL. The specificity of these mAbs was

Table 6
Reactivity of the mAbs with non-B cell and non-B cell lines

Code	Clone name	Granulo.	Mono.	K-562	HL-60	U-937	DCs	Platelets	RBC
B001	17.20	–	–	++	–	–	–	–	–
B002	18.12	ND	+	–	–	–	ND	ND	ND
B003	EM1.24	–	++++	++++	++++	++++	+++	–	–
B004	AS38	–	++++	++++	++++	++++	+++	–	–
B005	K5C1	–	+++	++++	++++	+++	+++	–	–
B006	SC3	+++	–	–	–	–	++++	+	+
B007	FH25	+	+++	–	+	–	–	–	–
B008	F821	++++	+++	+++	++	+	++	–	+
B009	HTL-131	–	++	++++	++++	+	+++	–	–
B010	HT220	ND	++	–	–	–	ND	ND	ND
B011	HT301	ND	++	–	–	–	ND	ND	ND
B012	HT305	++++	+	–	++	–	–	++	–
B013	HT306	–	++	–	–	–	++++	–	–
B014	HT307	ND	++	–	–	–	ND	ND	ND
B015	HT302	ND	++	–	–	–	ND	ND	ND
B016	IF11	–	–	++++	++++	++++	–	–	–
B017	8A12	–	+	+++	+++	++	–	–	–
B019	70513	ND	–	–	–	–	ND	ND	ND
B021	FMU60	ND	–	+	–	+	ND	ND	ND
B022	FMU76	ND	–	–	–	+	ND	ND	ND
B023	803-761	ND	+++	–	+	–	ND	ND	ND
B026	Vicky-1	–	–	–	–	–	–	–	–
B028	FMU-TAC12	ND	+++	++	–	++++	ND	ND	ND
B051	FMU-BCMA1	ND	–	+	–	+	ND	ND	ND
B053	SICD134L-2(4C12)	–	–	+	–	+	+	+++	–
B057	SICD137L-1F1	–	–	–	–	+	–	–	–
B058	9A5	–	–	++	–	+++	–	–	–
B059	F56	–	–	–	–	–	–	–	–
B060	F23	–	–	–	–	–	–	–	–
B061	F119	–	–	–	–	–	–	–	–
B062	1A1	–	–	–	–	–	–	–	–
B063	1C1	–	–	+	–	–	–	–	–

The cells were incubated with the different mAbs. Then cells were washed and incubated with biotinylated anti-mouse light κ chain followed by streptavidin-PE. A FITC-conjugated anti-rat IgG antiserum was used with rat mAbs. Results represent the percentage of positive cells: –, <20% of positive cells; +, 20–40% of positive cells; ++, 40–60% of positive cells; +++, 60–80% of positive cells; +++++, 80–100% of positive cells; ND: not done; granulo.: granulocytes; mono.: monocytes; DCs: dendritic cells; RBC: red blood cells.

confirmed with transfected cells (data not shown). During B cell development, we observed expression of EW1-2 cell-surface molecule from pro-B cells to plasma cells, with expression increasing after activation (Table 3). It was also highly expressed on T cells (Table 5). Although the staining of cell lines was identical with both antibodies, differences were observed in its reactivity with isolated tonsil B cells and NK cells (Tables 3 and 5). In tonsil sections stronger staining in the germinal center was detected with 8A12.

70513 (B019). This mAb recognized TRANCE (TNFSF11) and was reactive with the cell-surface of several B cells and B cell lines (Tables 3 and 4). 70513 mAb stained scattered cells in the interfollicular zone in tonsil sections (Table 7).

Vicky-1 (B026) and FMU-BCMA1 (B051). While these two mAbs recognizes BCMA (TNFSF17), Vicky-1 mAb showed very faint staining. Their reactivity was shown to be B cell restricted. Pre-B cells and mature B cells expressed low levels of the BCMA protein, while

highest levels of expression were found on immature B cell and plasma cell lines (Tables 3 and 4).

FMU-TAC12 (B028) and 1A1 (B062). Although these two mAbs recognized TAC1 (TNFSF13B), FMU-TAC12 mAb showed very faint staining. Low levels of TAC1 were detected on tonsil B cells and several B cell lines (Tables 3 and 4). While no up-regulation of expression could be detected after in vitro B cell activation (Table 3), strong expression on EBV-transformed B cells was observed (Table 4). Unexpectedly, FMU-TAC12 mAb reacted with some Burkitt lymphoma cell lines that were negative for 1A1 mAb (Table 3).

SICD134L-2(4C12) (B053). This mAb recognized OX40L (TNFSF4). The specificity of this mAb was confirmed with transfected cells (data not shown). Strong expression of OX40L was observed on isolated B cells and platelets (Tables 3 and 6). CD34⁺ bone-marrow cells expressed detectable levels of this molecule (Table 5). During B cell development, all B cells expressed this molecule, the strongest expression occurring from the B immature

CD307 (IRTA-2 or FcRH5) belongs to the newly designated Fc receptor homolog family [31,32]. This molecule, together with CD267 (TACI) and CD269

(BCMA), were the only molecules analyzed in this study that proved to be B cell restricted. Thus, far, relatively few B cell-associated markers have been shown to be lineage-restricted [8]. Four of these molecules form part of the pre-B and B cell receptor complexes (CD79a, CD79b, CD179a, and CD179b), while the rest are expressed by different B cell subsets, namely CD19, CD20, CD21, and CD22 [9]. Heretofore, prevailing opinion has suggested that all B cell restricted molecules had already been described. In fact, it is quite possible that the number of known cell surface molecules exhibiting a B cell restricted expression pattern may well increase during the next few years. For example, most of the new Fc receptor homolog family members that have recently been cloned, including CD307, seem to be expressed only by B cells [33]. In this study, we have also sought to address the expression levels of these B cell-associated molecules on other leukocytes. Most mAbs that identify cell-surface molecules were not lineage specific, they were expressed not only on B cells, but also on several leukocytes and lymphocyte subsets. The importance of cross-lineage comparison is based on the realization that many molecules, even one like CD4, which was thought to be lineage-restricted, are actually expressed by multiple cell lineages. It is therefore of crucial importance to increase our knowledge of the expression patterns for a better insight into these molecules.

Half of the mAbs submitted against unknown molecules were able to recognize MHC class II molecules. The targets for the remaining mAbs could not be determined. At least four mAbs, including 37.20 (B001), SC2 (B006), FH25 (B007), and FB21 (B008) could apparently identify new B cell-associated molecules selectively expressed on B cell subpopulations. Cloning the molecules that these mAbs recognize will be an important goal of future studies.

In conclusion, the availability of mAbs directed against novel B cell surface molecules will have not only broad implications for B cell biology, but also for development of new diagnostic and therapeutic tools.

References

- [1] A.N. Barclay, M.H. Brown, S.K.A. Law, A.J. McKnight, M.G. Tomlinson, P.A. van der Merwe, *The Leucocyte Antigen Facts-Book*, Academic Press, Oxford, 1997.
- [2] D.Y. Mason, P. Andre, A. Bensussan, C. Buckley, C. Givin, E.A. Clark, M. de Haas, S. Goyert, M. Hadam, D. Hart, V. Horejsi, S. Meuer, J. Morrissey, R. Schwartz-Albiez, S. Shaw, D. Simmons, M. Ugucioni, E. van der Schoot, E. Vivier, H. Zola, CD antigens 2001, *J. Leukoc. Biol.* 70 (2001) 685–690.
- [3] H. Zola, B. Swart, L. Boumsell, D.Y. Mason, Human leukocyte differentiation antigen nomenclature: update on CD nomenclature—Report of IUIS/WHO subcommittee, *J. Immunol. Methods* 275 (2003) 1–8.
- [4] H. Zola, B. Swart, J. Nicholson, B. Aasted, A. Bensussan, L. Boumsell, C. Buckley, G. Clark, K. Drbal, P. Engel, S. Goyert, D. Hart, D. Haylock, V. Horejsi, C. Isacke, P. Macardie, F. Malavasi, D.Y. Mason, J. Morrissey, D. Olive, A. Sanhuccler, S.P. Schlossman, R. Schwartz-Albiez, P. Simmons, T.F. Tedder, M. Ugucioni, H. Warren, CD Molecules: human cell differentiation molecules, *Blood* (2005) in press.
- [5] M.J. Jarosinski, R. Heller, *Flow Cytometry Protocols, Methods in Molecular Biology* 91, Humana Press, Totowa, 1998.
- [6] R.R. Hardy, K. Hayakawa, B cell development pathways, *Annu. Rev. Immunol.* 19 (2001) 595–621.
- [7] E.A. Clark, P.J.L. Lane, Regulation of human B-cell activation and adhesion, *Annu. Rev. Immunol.* 9 (1991) 97–127.
- [8] D.Y. Mason, M. Jones, D.L. Hardie, G. van Schijndel, G.D. Johnson, R. van Lier, I.C.M. MacLennan, Blind panel report, in: T. Kishimoto, H. Kikutani, A.E.G.K. von dem Borne, S.M. Goyert, D.Y. Mason, M. Miyasaka, L. Moretta, K. Okumura, S. Shaw, T.A. Springer, K. Sugamura, H. Zola (Eds.), *Leucocyte Typing VI*, Garland Publishing, New York, 1997, pp. 206–229.
- [9] E.A. Clark, D.Y. Mason, B-cell section report, in: P. Andre, A. Bensussan, C. Buckley, C. Givin, E.A. Clark, M. de Haas, S. Goyert, M. Hadam, D. Hart, V. Horejsi, Y. Jones, S. Meuer, J. Morrissey, R. Schwartz-Albiez, S. Shaw, D. Simmons, L. Turai, M. Ugucioni, E. van der Schoot, E. Vivier, H. Zola (Eds.), *Leucocyte Typing VII*, Oxford University Press, Oxford, 2002, pp. 81–89.
- [10] J. Ishikawa, T. Kishino, H. Tomizawa, B.O. Lee, Y. Kobune, J. Inazawa, K. Oritani, M. Itoh, T. Ochi, K. Ishihara, T. Hirano, Molecular cloning and chromosomal mapping of a bone-marrow stromal cell-surface gene, *Bst2*, that may be involved in Pre-B-cell growth, *Genomics* 26 (1995) 527–534.
- [11] T. Ohtomo, Y. Sugamata, Y. Ozaki, K. Ono, Y. Yoshimura, S. Kawai, Y. Koishihara, S. Ozaki, M. Kozaka, T. Hirano, M. Taniuchi, Molecular cloning and characterization of a surface antigen preferentially overexpressed on multiple myeloma cells, *Biochem. Biophys. Res. Commun.* 258 (1999) 583–591.
- [12] T. Goto, S.J. Kennel, M. Abe, M. Takishita, M. Kosaka, A. Solomon, S. Salto, A novel membrane antigen selectively expressed on terminally differentiated human B-cells, *Blood* 84 (1994) 1922–1930.
- [13] S. Ozaki, M. Kosaka, Y. Wakahara, Y. Ozaki, M. Tsuchiya, Y. Koishihara, T. Goto, T. Matsumoto, Humanized anti-HM1.24 antibody mediates myeloma cell cytotoxicity that is enhanced by cytokine stimulation of effector cells, *Blood* 93 (1999) 3922–3930.
- [14] E.J. Evans, L. Hens, L.M. Sparks, T. Dong, C. Retiere, J.A. Fennelly, R. Manso-Sanchez, J. Powell, V.M. Braud, S.L. Rowland-Jones, A.J. McMichael, S.J. Davis, The T cell surface—How well do we know it? *Immunity* 19 (2003) 213–223.
- [15] A.H. Sharpe, O.J. Freeman, The B7-CD28 superfamily, *Nat. Rev. Immunol.* 2 (2002) 116–126.
- [16] S. Charrin, F. Le Naour, M. Oualid, M. Billard, G. Faure, S.M. Hanash, C. Boucheix, E. Rubinstein, The major CD9 and CD81 molecular partner—identification and characterization of the complexes, *J. Biol. Chem.* 276 (2001) 14329–14337.
- [17] S. Charrin, F. Le Naour, V. Lahas, M. Billard, J.P. Le Caer, J.F. Emile, M.A. Petit, C. Boucheix, E. Rubinstein, EWI-2 is a new component of the tetraspanin web in hepatocytes and lymphoid cells, *Biochem. J.* 373 (2003) 409–421.
- [18] C.S. Stipp, T.V. Kolesnikova, M.E. Hemler, EWI-2 is a major CD9 and CD81 partner and member of a novel Ig protein subfamily, *J. Biol. Chem.* 276 (2001) 40545–40554.
- [19] K.L. Clark, Z.H. Zeng, A.L. Langford, S.M. Bowen, S.C. Todd, PGRL is a major CD81-associated protein on lymphocytes and distinguishes a new family of cell surface proteins, *J. Immunol.* 167 (2001) 5115–5121.
- [20] E. De Leenbeer, G.S. Mueller, K. Vanderkerken, F.J. Croucher, Evidence of a role for RANKL in the development of myeloma bone disease, *Curr. Opin. Pharmacol.* 4 (2004) 340–346.
- [21] P.J. Croucher, C.M. Shipman, J. Lippitt, M. Perry, K. Asosingh, A. Hijzen, A.C. Brabbs, E.J.R. van Beek, I. Holen, T.M. Skerry, C.R.

- Dunstan, C.R. Russell, B. Van Camp, K. Vanderkarken, Osteoprotegerin inhibits the development of osteolytic bone disease in multiple myeloma, *Blood* 98 (2001) 3534–3540.
- [22] F. Mackay, J.L. Browning, BAFF: a fundamental survival factor for B cells, *Nat. Rev. Immunol.* 2 (2002) 465–475.
- [23] D.T. Avery, S.L. Kallied, J.L. Elyard, C. Ambrose, S.A. Bialer, M. Thien, R. Brink, F. Mackay, P.D. Hodgkin, S.G. Tangye, BAFF selectively enhances the survival of plasmablasts generated from human memory B cells, *J. Clin. Invest.* 112 (2003) 286–297.
- [24] B.P. O'Connor, V.S. Raman, L.J. Erickson, W.J. Cook, L.K. Weaver, C. Ahonen, L.L. Lin, G.T. Mantchev, R.J. Bram, R.J. Noelle, BCMA is essential for the survival of long-lived bone marrow plasma cells, *J. Exp. Med.* 199 (2004) 91–97.
- [25] F. Mackay, S.G. Tangye, The role of the BAFF/APRIL system in B cell homeostasis and lymphoid cancers, *Curr. Opin. Pharmacol.* 4 (2004) 347–354.
- [26] H. Wang, S.A. Marsters, T. Baker, B. Chan, W.P. Lee, L. Fu, D. Tumas, M.H. Yan, V.M. Dixit, A. Ashkenazi, I.S. Grewal, TACI-ligand interactions are required for T cell activation and collagen-induced arthritis in mice, *Nat. Immunol.* 2 (2001) 632–637.
- [27] J.A. Goad, S.R. Dillon, S. Mudri, J. Johnston, A. Littau, R. Ruque, M. Rixon, O. Schou, K.P. Foley, H. Haugen, S. McMillen, K. Waggle, R.W. Schreckhise, K. Shoemaker, T. Vu, M. Moore, A. Grossman, C.H. Clegg, TACI-Ig neutralizes molecules critical for B cell development and autoimmune disease: impaired B cell maturation in mice lacking BLyS, *Immunity* 15 (2001) 289–302.
- [28] L.G. Ng, A.P.R. Sutherland, R. Newton, F. Qian, T.G. Casboro, M.L. Scott, J.S. Thompson, J. Wheway, T. Chikanova, J. Groom, L.J. Sutton, C. Xin, S.G. Tangye, S.L. Kallied, F. Mackay, C.R. Mackay, B cell-activating factor belonging to the TNF family (BAFF)-R is the principal BAFF receptor facilitating BAFF costimulation of circulating T and B cells, *J. Immunol.* 173 (2004) 807–817.
- [29] W.R. Godfrey, F.F. Fagnoni, M.A. Harara, D. Buck, E.G. Engleman, Identification of a human OX-40 ligand, a costimulator of CD4+ T-cells with homology to tumor-necrosis-factor, *J. Exp. Med.* 180 (1994) 757–762.
- [30] E. Stuber, W. Strober, The T cell B cell interaction via OX40-OX40L is necessary for the T cell-dependent humoral immune response, *J. Exp. Med.* 183 (1996) 979–989.
- [31] G. Hatzivassiliou, I. Miller, J. Takizawa, N. Palaniyandy, P.H. Rao, S. Iida, S. Tagawa, M. Tanikawa, J. Russo, A. Neri, G. Cattoretti, R. Clynes, C. Mendelsohn, R.S.K. Chaganti, R. Dalla-Favera, IRTA1 and IRTA2, novel immunoglobulin superfamily receptors expressed in B cells and involved in chromosome 1q21 abnormalities in B cell malignancy, *Immunity* 14 (2001) 277–289.
- [32] I. Miller, G. Hatzivassiliou, G. Cattoretti, C. Mendelsohn, R. Dalla-Favera, IRTAs: a new family of immunoglobulin like receptors differentially expressed in B cells, *Blood* 99 (2002) 2662–2669.
- [33] R.S. Davis, G. Dennis, M.R. Odom, A.W. Gibson, R.P. Kimberly, P.D. Burrows, M.D. Cooper, Fc receptor homologs: newest members of a remarkably diverse Fc receptor gene family, *Immunol. Rev.* 190 (2002) 123–136.

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